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Im Auftrag

For the President of the European Patent Office

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Method and test-kit for the detection and quantification of low levels of expressed active enzymes and/or microorganisms

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Method and Test-Kit for the Detection and Quantification of Low Levels of Expressed Active Enzymes and/or Microorganisms

Field of the invention

The invention relates to novel methods for the detection and or quantification of low levels of expressed active enzymes of interest, and/or consequently the microorganisms producing enzymes, and the application of said method in the cosmetic field, food industry, industrial and institutional cleanliness. More specifically the invention is concerned with methods for the measurement of enzymes of microorganisms obtained directly from their habitat without previously culturing the microorganisms.

Background of the invention

Specific enzymes of microorganisms, e.g. lipases/esterases on surfaces such as human skin or hair, are responsible for odour development, the development of acne or the generation of dandruff on the scalp. In order to avoid the development of axillary malodour or dandruff, deodorants and anti-dandruff shampoos often contain anti-microbial substances. These inhibit the growth of bacteria and thereby reduce the amount of enzymes responsible for the development of axillary malodour or the formation of dandruff.

Methods to evaluate the efficiency of anti-microbial substances of interest encompass the detection of the microorganisms or the enzymes, or the determination of the

enzymatic activity after contacting the enzyme or microorganism with said substances.

Enzyme expression can be measured indirectly by detection of the encoding mRNA using the RT-PCR method. In this procedure an RNA sample is copied into cDNA, which is then amplified by PCR. The amplified DNA fragments can be analysed e.g. by agarose gel electrophoresis and the amount of produced fragments is essentially proportional to the amount of target RNA and consequently to the amount of protein. This method, however, is costly, time consuming and the quantification is often not precise. Moreover, the PCR is only an indirect measurement of the expression level of a target enzyme. Additionally, this method does not give any information as to whether an enzyme is present in an active form.

Microorganisms of interest are typically detected by culturing on solid media until colonies are visible, or by sterility testing in liquid media. Other assays are based on immunoassays, or on PCR for detecting specific organisms.

The APIZYM system provides a method for microorganism detection through their enzyme activities (EP 1451775). However, the system sold by bioMérieux is based on a plastic strip holding a number of miniature biochemical test tubes including fermentation tests and enzymatic reactions (usually about twenty tests). The tubes containing enzymes substrates are inoculated with a suspension of culture. The reactions either produce directly a colour change or are revealed by the addition of reagents. Results are read and interpreted either by comparing the profile list included in the instruction manual or by computer analysis using APILAB

software. This system requires a culturing step, and is therefore time-consuming, laborious and costly.

The ATP bioluminescence assay allows for a sensitive detection and quantification of microorganisms. Microorganism levels are measured through the presence of ATP they contain. It was found that the firefly produces its characteristic glow by utilizing ATP in a reaction with the luciferase enzyme and luciferin substrate to release energy as light. The light produced by the reagents can then be measured (A. Lundin, Analytical applications of bioluminescence: The firefly system, pp. 44-74, in "Clinical and Biochemical Luminescence", Eds. L.J. Kricka and T.J.N. Carter, Marcel Dekker, Inc., New York, 1982). However, this method is not specific since ATP is a universal energy molecule common to all living cells and organisms and is therefore present not only in microorganisms but also on many residues and surfaces such as the skin.

EP 0 786 247 describes a sustained-release aromatic, comprising a perfume derivative as an active component. Microorganisms present on skin can split said perfume derivative. Furthermore, a detection method for the microorganisms by means of measuring the released aroma of the split perfume derivatives is also claimed. However, the detection of a released aroma is not convenient and provides only semi-quantitative results with regard to the presence and amount of a given enzyme.

WO 01/92563 generally describes a method for detecting and quantifying a chemical transformation and more specifically an enzymatic transformation in a sample. The method uses a stable substrate, which is enzymatically transformed and

thereafter the transformed substrate is measured. This method is not used for the detection and quantification of the expression of a specific enzyme or the detection or quantification of microorganisms expressing said enzyme, and which are located on an ecosystem or a surface, e.g. the skin.

Efficiency testing of raw materials for personal care products and cosmetic formulations are a main issue in the industry. The efficiency testing of deodorants for example is generally evaluated by three approaches: in vitro microbiological evaluation, chemical analysis and sensory evaluation of the odour. However, in case of evaluation of substances affecting axillary malodour, the chemical analysis method is limited by the complex and variable nature of said malodour. Only single chemicals can be detected, which are known to be part of the axillary malodour.

EP 0 666 732 describes new antimicrobial substances for deodorants and *in vitro* tests for their efficiency. The antimicrobial activity against single germs like *Corynebacterium xerosis* is investigated. This test is time consuming, since it takes up to 48 h. Moreover, the test requires that germs be cultivated first and is therefore expensive. Additionally, the results are sometimes falsely negative.

EP 1 250 842 describes new antimicrobial substances for deodorants and their efficiency testing *in vivo* by sensory evaluation. Sniff tests, however, are cost intensive, because specially trained experts are needed. Additionally, the quantification of these results can be difficult and imprecise.

A new approach to the assessment of deodorants deals with the counting of the axillary germ population by cultivation of said germs with and without treatment of the armpits. This method is efficient, but also time consuming, because the incubation of the germs takes 2 to 4 days (A. R. Cox, J. Soc. Cosmet. Chem. 38, 223-231 (1987); C. Fearnley, A. R. Cox, Int. J. Cosmet. Science 5, 91-109 (1983).)

Axillary malodour is mainly produced by skin microflora having lipase activity, especially by corynebacteria. (P.J. Rennie, et al., Br. J. Dermatol. 124, 596-602 (1991); J.J. Leyden et al., J. Invest. Dermatol. 77, 413-416 (1981); G. Pablo, et al., J. Invest. Dermatol. 63, 231-238 (1974).)

The application of certain antimicrobial substances to the skin is directly linked to the reduction of lipase activity. Hence, the efficiency of those antimicrobial agents and/or deodorant formulations correlates with the reduction of lipase activity, or more precisely, with the effect on the viability of microorganisms expressing said lipases.

Furthermore, anti-dandruff formulations are usually evaluated by determination of the antimicrobial activity against *Malassezia furfur* or by clinical trials. Both methods are time consuming and expensive.

Disclosure of the invention

In the light of the foregoing, wherein the methods for determination of expressed enzymes of interest and the microorganisms harbouring the same are generally tedious, costly and time consuming, it would be desirable to provide

methods for detecting and quantifying expressed enzymes of interest and the microorganisms expressing the same that do not suffer from the above mentioned drawbacks.

Definitions:

In the following, several terms used throughout the description will be explained:

Enzyme of interest

Any expressed enzyme that is of interest in the field of the invention, i.e. any expressed enzyme that acts on substances used in the field of cosmetics, food industry, industrial and institutional cleanliness and related fields. Preferred examples for such enzymes are esterases, and particularly lipases.

Substance of interest

Any substance, composition or formulation that exerts an effect on an enzyme or a microorganism. The substance, composition or formulation can also acquire an activity after reacting with the expressed enzyme or microorganism. The active substance can be for example a drug, an antimicrobial agent, or a cosmetically active substance. The antimicrobial agent can inhibit the viability of microorganism and thereby reduces the catalytic activity of said enzyme.

Sampling tool

Sampling tools used within the meaning of the present invention are all those generally known to a person skilled in the art, specifically those which can rinse off and collect fluid, which suck off fluid and/or solid matter, like swabs, syringes, or smear tests or tape strippings.

In a preferred embodiment, the enzyme substrate used to measure the hydrolysis of ester corresponds to 2-hydroxy-4-p-nitrophenoxy-butyl decanoate. The assay is based on the enzymatic transformation of the substrate upon catalytic activity followed by a two-steps degradation pathway including an oxidation and a ß-elimination reaction which allows the release of the chromogenic signal.

Direct detection/quantification

As used herein, this term means, that the microorganism is not cultured *in vitro* before a sample suspected of containing the microorganism expressing an enzyme of interest is subjected to the determination of the enzymatic activity.

Detailed description of the invention

The present invention provides a simple method for a highly sensitive, cheap, direct, fast detection and quantification of the presence of the expression of a specific enzyme of interest or a microorganism through the measurement of the enzymatic activity. The method indirectly allows for detection and/or quantification of a microorganism harbouring said enzyme.

In one embodiment, the present invention encompasses a method for the detection and/or quantification of an expressed enzyme of interest. Said method comprises the steps of:

a) collecting a sample suspected of containing an enzyme of interest,
b) incubating said sample with at least one substrate transformable by said enzyme, and

c) detecting the transformed substrate.

Preferably, the method for the detection and/or quantification of an expressed enzyme of interest, or of the microorganism expressing said enzyme of interest, is a direct method. That is, it does not involve the culturing of said microorganism before detection and/or quantification of the enzyme. Said method comprises the steps of:

a) collecting a sample suspected of containing a microorganism expressing said microbial enzyme of interest,

b) incubating said sample with at least one substrate transformable by said enzyme of interest,

c) detecting the transformed substrate.

The methods according to the present invention can be performed in a clearly reduced time span relative to the state of the art; usually 1 to 3h, preferably 1.5h to 2.5h are sufficient.

In a preferred embodiment of the invention, the sample containing the microorganism is derived from a bodily surface, such as skin or hair of an animal, preferably a human being. Preferably, the surface is the skin, preferably of armpits or the scalp of humans. Most preferably, the enzyme is a microbial enzyme derived from an organism that is present on the skin or hair.

In another preferred embodiment, the microorganism is a bacterium, fungus, virus or yeast, more preferably a bacterium or a fungus present on the skin or hair of an animal, preferably a human being. Exemplary microorganisms

according to the present invention can be *Staphylococci*, *Corynebacteria*, *Micrococci* and *Propionibacteria*.

Preferably, the microbial enzyme of interest is an esterase, more preferably a lipase.

In still another embodiment of the invention, the substrate transformable by the enzyme of interest to release a detectable product is an ester. More preferably, the ester is a C3 bis C18 ester, preferably a 2-hydroxy-4-p-nitrophenoxy-butyl carboxylic acid ester, and particularly preferred it is 2-hydroxy-4-p-nitrophenoxy-butyl decanoate.

As axillary odor is closely related to the lipase activity of armpit germs, the efficiency of compounds in cosmetic formulations, e.g. deodorants or shampoos can be easily measured by the method of the invention. To this end, the presence of an expressed enzyme of interest is evaluated; this is done with and without previous application of a cosmetic composition comprising a substance of interest to the site the sample suspected of containing an enzyme of interest is derived from.

Preferably, the method of the invention relates to the evaluation of an inhibitory effect of a substance of interest on a microorganism expressing an enzyme of interest, whose activity is to be determined.

Advantageously, said method comprises the following steps:
a) contacting the microorganism expressing an enzyme of interest with a substance of interest,
b) collecting a sample suspected of containing said enzyme of interest,
c) incubating said sample with at least one transformable substrate,
d) detecting the transformed substrate,

e) comparing the level of transformed substrate to the level of transformed substrate that is obtained without performing the step (a).

That is, the method may be used to compare the efficiency of a test substance of interest that is brought into contact with microorganisms, with a negative control test, wherein the test substance is not applied to the microorganisms. The effectiveness of the test substance can be evaluated on the basis of the difference between the effect exerted on the microorganisms that have been, or have not been treated with the test substance.

For example, if a test substance inhibits the growth of microorganisms, the activity of enzymes expressed by these microorganisms generally also tends to decrease. When the activity of a selected enzyme expressed by the microorganisms, which have been treated with the test substance is compared with the enzyme activity of microorganisms that have not been treated with the test substance, and therefore have not experienced a growth inhibition, the determination of the efficiency of the test substance is possible. In principle this can be achieved by any analytical method, preferably UV or IR spectrometry or fluorescence.

A preferred application of the above explained test method, is the evaluation of the efficiency of antimicrobial substances.

In a particularly preferred method of the invention, the substance to be analysed is selected from the group consisting of anti-acne compounds, anti-acne compositions, deodorant compounds, deodorant compositions, compounds of a shampoo, or shampoo compositions, preferably of anti-dandruff shampoos.

The present invention also relates to a kit for the evaluation of the expression of an enzyme of interest or microorganism, said kit comprising:

a) a sampling tool
b) a substrate transformable by an expressed enzyme
c) optionally reagents for the detection of the transformable substrate.

In a preferred embodiment of the invention, the kit is used for the evaluation of an inhibitory effect of a substance of interest on a microorganism, e.g. a bacterium or fungus, said kit comprising:

a) a sampling tool,
b) a substrate transformable by a microbial enzyme expressed by said microorganism,
c) optionally reagents for the detection of the transformable substrate.

In still another preferred embodiment, the reduction of lipase activity can be measured easily with the described method or kit. Thus, the method and the kit are used for confirming the presence of an active microbial enzyme, e.g. a lipase/esterase on the skin, wherein the enzyme is indicative for the presence and quantity of microorganisms expressing said enzyme.

In the following examples, it is furthermore shown that the reduction in lipase activity correlates with the total count of bacteria.

Examples

Example 1:

Microorganisms or enzymes were sampled directly on the skin using a swab impregnated with aqueous acetate buffer (0.1 M, pH 5.6) containing 0.1% w/v of Triton X100. The sampling surfaces were chosen to be approximately 10 cm^2. The time of sampling was approximately 1 minute.

After sampling, the swab was immersed in 200 µl of a 2-hydroxy-4-p-nitrophenoxy-butyl decanoate (C10-HpNPB) substrate solution (16 µl of a 20 mM stock solution in DMSO), in aqueous acetate buffer (0.1 M, pH 5.6, 184 µl). The enzymatic reaction was performed at 37°C for 1.5 hours.

After incubation, the swab was removed from the reaction mixture, and 100 µl were transferred to another vial for detection of transformed substrate: 40 µl of BSA (from an aqueous stock solution at 50 g/L), 4 µl of $NaIO_4$ (from an aqueous stock solution at 100 mM) and 40 µl of Na_2CO_3 (from an aqueous stock solution at 200 mM) were added and let react at room temperature for 10 minutes. The sample was centrifuged at 16,000 g for 15 minutes, the supernatant was transferred to a 96-well-microtiterplate, and the hydrolysis of the CLIPS-O™ substrate was quantified by measuring the optical density at 414 nm using a Spectramax 190 microtiterplate spectrophotometer (Molecular Devices). [D. Lagarde et al., Org. Process Res. Dev., 6, pp. 441 (2002)].

Results:

In a first test series the mode of action of glyceryl esters as modulator substances was evaluated. Choosing a 2-hydroxy-4-p-nitrophenoxy-butyl decanoate (C10-HpNPB) as control substrate for the quantification, it was shown that said fatty acid ester is cleaved by enzymes from microbes sampled from the armpit and other surfaces of the body using the method described above. The results of said test are described in the below table.

	Arm	Armpit	Forehead	Scalp
Volunteer A	0.116	0.624	0.321	0.157
Volunteer B	0.063	0.267	0.186	0.389
Volunteer C	0.077	0.185	0.108	0.082
Volunteer D	0.091	0.260	0.293	0.157
Volunteer E	0.057	0.047	0.164	0.164
Control (average of 4 values): 0.049				

Results expressed in OD at 414nm
Control: Blank experiment without sampling

As expected, due to the usually low concentration of microorganisms on the arms, the activities recorded are very low. However, in the case of samples from the armpit, forehead and scalp the average values are higher. However, differences observed between the volunteers are probably due to the efficiency of their applied own personal care products.

Example 2:

Lipase activity

In a second series polyglyceryl-3 caprylate (Tego Cosmo P813, Goldschmidt) as modulatory substance was chosen as the deodorant active. The deodorant was applied to one armpit of a test person (2% Laureth-23 (TEGO Alkanol L 23P, Goldschmidt), 0.5% polyglyceryl-3 caprylate, 97.5% water). The other armpit was treated with a control (2% Laureth-23, 98% water) and was used as a reference. In the evening the test persons had a shower. Then deodorant and control were applied. In the morning deodorant and control were used again. At noon the reduction of bacteria count relative to the reference was measured using the method and kit described above. In 7 of 9 cases a significant reduction of lipase activity was found:

	Deodorant	Control	Control - Deodorant
Person A	0.51	1.04	0.53
Person A	0.11	0.29	0.18
Person A	0.27	1.05	0.78
Person B	0.67	0.72	0.05
Person B	0.12	0.18	0.06
Person B	0.13	0.75	0.62
Person C	0.51	1.04	0.53
Person C	0.48	1.11	0.63
Person C	0.45	1.10	0.65

The results were compared to a conventional sniff test and an antimicrobial *in vitro* test.

Sniff test

20 test persons washed their axilla. The odor was evaluated by three experts. Then a polyglyceryl-3 caprylate solution (2% Laureth-23 (TEGO Alkanol L 23P, Goldschmidt), 0.3% polyglyceryl-3 caprylate, 97.7% water) was applied to one armpit of each test person. The other armpit was left untreated and was used as a reference. The axilla odor was evaluated after 6 and 24h. A significant improvement of axillary odor was found compared to the untreated axilla.

The results correspond to the results gained using the method and kit described above.

In vitro microbiological evaluation

An EuAB test for antimicrobial activity was performed with *Corynebacterium xerosis* (DSM 20743), *Staphylococcus epidermidis* (DSM 3269) and *Candida albicans* (ATCC 10231). As culture medium CSL (Casein peptone-soybean meal peptone solution), CSA (Casein peptone-soybean meal pepton agar) and Sabouraud-glucose broth/agar was used.

As dilution liquid NaCl-peptone buffer solution with inactivator (3% Tween 80, 0.3% lecithin, 0.1% histidine and 0.5% Na thiosulfate) was used. Polyglyceryl-3 caprylate was tested in 0.3% (in CSL) concentration. The test solution was prepared one day before investigation. For this, 100 ml of CSL was heated to 60°C in a water bath. 0.3 g was weighed into 100 ml of CSL at 60°C. The preparations were shaken vigorously by hand and left overnight at 30°C in an

incubator. *Corynebacterium xerosis* was cultivated over 3 to 4 days. Other microbes were isolated in broth or by elutriation. For each test microbe, 20 ml of each test solution were introduced into sterile 50 ml brown glass bottles with glass beads and contaminated with 0.2 ml of microbe suspension. As controls, 20 ml of CSL were carried over per test microbe without sample.

The contaminated samples were shaken for 3 min on a shaking machine and kept in an incubator at 30°C until removed. At the removal points (1, 2, 3, 24 and 48 hours) 1 ml was taken from each preparation and transferred to in each case 9 ml of NaCl-peptone buffer solution with inactivator and the colony number was determined. The 0 hours values given were the colony numbers of the test microbe suspension used taking into consideration the 10^{-2} dilution upon sample contamination.

The results are given in Figure 1 enclosed herewith. Also shown is the microbe population of an active-ingredient-free blind sample as control value after incubation for 24 hours. A reduction of all bacteria count from 10^7 to < 270 could be found after 24h.

This *in vitro* method also proves the antimicrobial efficiency of polyglyceryl-3 caprylate. Therefore all three methods show concurrent results. But the test method described in this invention is by far the fastest and easiest of all three.

Claims

1. A method for the detection and/or quantification of the expression of an enzyme of interest, comprising the steps:
a) collecting a sample suspected of containing said enzyme,
b) incubating said collected sample with at least one substrate transformable by said enzyme,
c) detecting and optionally quantifying the transformed substrate.

2. The method according to claim 1, wherein the expressed enzyme is located on the surface of an ecosystem.

3. The method according to claim 1, wherein said enzyme is produced by a microorganism.

4. A method for the detection and/or quantification of a microorganism of interest, comprising the steps of:

a) collecting a sample suspected of containing said microorganism of interest,
b) incubating said collected sample with at least one substrate transformable by an enzyme produced by said microorganism,
c) detecting and optionally quantifying the transformed substrate.

5. A method for detecting and/or quantifying a modulatory effect of a substance of interest on the growth of a microorganism suspected to be present in an ecosystem, and suspected of producing an enzyme of interest, comprising the steps of:
a) administration of said substance to said ecosystem
b) collecting a sample of said ecosystem suspected of containing said microorganism producing said enzyme,
c) incubating said collected sample with at least one substrate transformable by said enzyme,

d) detecting and optionally quantifying the transformed substrate.

6. The method according to any of the previous claims, wherein the ecosystem is a surface of an animal body, including a human being.

7. The method according to any of the previous claims, wherein the animal is human.

8. The method according to any of the previous claims, wherein the surface is skin or hair.

9. The method according to any of the previous claims wherein the microorganism is a bacterium or a fungus.

10. The method according to any of the previous claims, wherein the enzyme is an esterase.

11. The method according to any of the previous claims, wherein the enzyme is a lipase.

12. The method according to any of the previous claims, wherein the substrate is an ester.

13. The method according to any of the previous claims, wherein the substrate is 2-hydroxy-4-p-nitrophenoxy-butyl carboxylic acid ester.

14. The method according to any of the previous claims, wherein the substrate is 2-hydroxy-4-p-nitrophenoxy-butyl decanoate.

15. The method according to any of the previous claims, wherein the transformed substrate is directly detectable, or it is detectable after at least one additional step following the enzymatic step.

16. The method of claims 4 to 15, wherein the amount of transformed substrate is compared with an amount of transformed substrate obtained in at least one control.

17. The method according to claims 5 to 16, wherein the control is either performed at the same time, before or later than the test for the detection and/or quantification of a modulatory effect of substance of interest.

18. The method according to claims 5 to 17, wherein the substance of interest is an antimicrobial substance.

19. The method according to claims 5 to 18, wherein the substance of interest is an anti-acne composition, a deodorant or a shampoo, preferably an anti-dandruff shampoo.

20. The method according to any of the previous claims, wherein the collected sample is incubated with the transformable substrate without prior cultivation.

21. A kit for the detection and/or quantification of the expression of an enzyme of interest, said kit comprising:
a) a sampling tool,
b) a substrate transformable by an enzyme of interest,
c) optionally reagents for the detection of the transformable substrate.

22. A kit for the detection and/or quantification of a modulator effect of a substance of interest as depicted in any of the methods of the previous claims, said kit comprising:

a) a sample tool
b) a substrate transformable by an expressed enzyme
c) optionally reagents for the detection of the transformable substrate.

Abstract

The present invention provides methods and test-kits for rapidly detecting and/or quantifying low levels of expressed active enzymes or microorganisms. In particular, the methods and kits can be used e.g. in the field of cosmetics, where the activities of (microbial) enzymes, which are responsible for malodour or dandruff, are detected as an indicator for the presence of expressed enzymes or microorganisms. Furthermore, the effect on substances acting on the causative microbes can be determined rapidly, easily and at low cost.

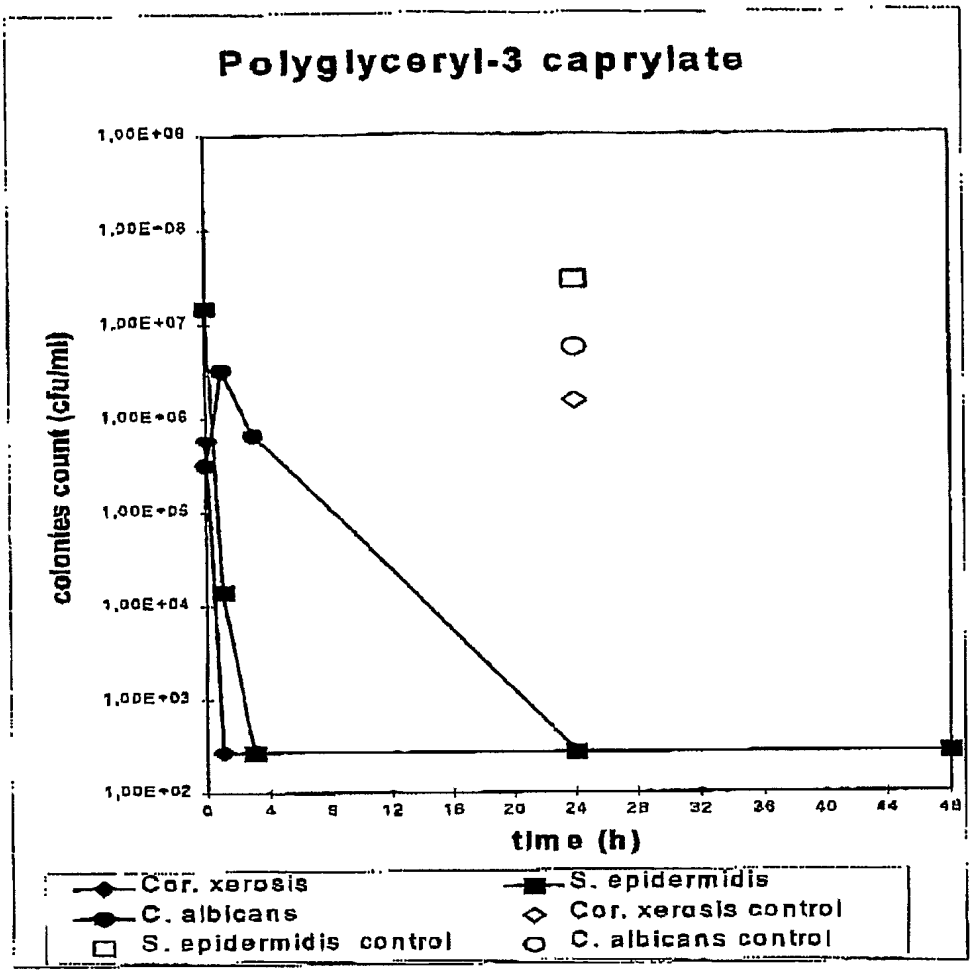
Polyglyceryl-3 caprylate
colonies count (cfu/ml)
1,00E+09
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
0
4
8
12
16
20
24
28
32
36
40
44
48
time (h)
Cor. xerosis
C. albicans
S. epidermidis control
S. epidermidis
Cor. xerosis control
C. albicans control

Fig. 1